

Functional binding of secreted molecules to heparan sulfate proteoglycans in *Drosophila*

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Heparan sulfate proteoglycans (HSPGs) are associated with the cell surface and covalently linked to a small number of long unbranched chains of repeating disaccharides. Numerous biochemical studies of these extracellular matrix molecules have implicated them in a variety of biological phenomena, in particular cell–cell interactions. Recent genetic studies in *Drosophila* have begun to clarify the function of HSPGs *in vivo* and recent findings have implicated HSPGs in Wnt, Hedgehog, fibroblast growth factor and transforming growth factor- β signaling pathways during development.

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Abbreviations

Dally	division abnormally delayed
Dpp	Decapentaplegic
FGF	fibroblast growth factor
GAGs	glycosaminoglycans
Hh	Hedgehog
HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
Sgl	Sugarless
Sfl	Sulfateless
Ttv	Tout-velu
UDP	uridine diphosphoglucose
Wg	Wingless

Introduction

Extracellular components exert their effects by binding to specific cell-surface receptors. In many instances, specific ligand–receptor interactions involve heparan sulfate proteoglycans (HSPGs). These large macromolecules are found abundantly at the cell surface and form part of the extracellular matrix. A proteoglycan (PG) is defined by the attachment of glycosaminoglycans (GAGs), which are sugars composed of long unbranched chains of repeating disaccharides, to specific serine residues on a core protein [1]. HSPGs are a subclass of PGs that carry sulfated GAG chains. The transmembrane syndecans and glycosylphosphatidyl inositol (GPI)-linked glypicans represent the two main cell-surface HSPGs, and most of the heparan sulfate (HS) at the cell surface is linked to these two classes of proteins (Figure 1).

Many biochemical studies and cell-culture assays have suggested that HSPGs interact with various growth factors, growth-factor-binding proteins, extracellular proteases, protease inhibitors and extracellular matrix molecules

(reviewed in [2,3*]). In particular, HSPGs have been proposed to act as coreceptors for a variety of secreted proteins, including fibroblast growth factor (FGF) and members of the Wnt, transforming growth factor- β (TGF- β) and Hedgehog (Hh) families [4–7].

Although biochemical data have provided ample examples of how HSPGs can modulate specific ligand–receptor interactions, it is only recently that *in vivo* evidence has emerged in both *Drosophila* and mice. In this review, we highlight recent *Drosophila* genetic studies that demonstrate the importance of HSPGs in specific signaling pathways during development. For recent reviews of the role of HSPGs in mice see [8,9].

Core proteins and enzymes involved in HSPG biosynthesis

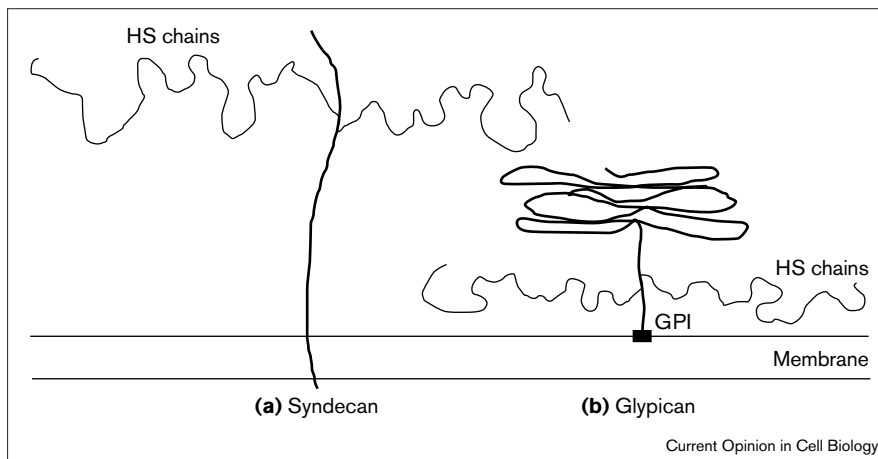
Genetic analyses of HSPGs have identified two gene classes that are involved in their biosynthesis. The first class encodes the core proteins onto which HS is synthesized. For example, in *Drosophila*, a single *syndecan* gene, two *glypican* genes (*dally* and *dally-like*), and a *perlecan* homologue have been characterized. So far, functional data are only available for the glypican family members (see below).

The second class contains the genes involved in the synthesis and modification of HS. These include the enzymes required for the biosynthesis of the sugars, the sugar transporters, and the enzymes involved in HS biosynthesis. At least 14 biochemical steps contribute to the synthesis of HS chains (see [10]). Precursor HS chains are initially synthesized in the Golgi as nonsulfated copolymers attached to HSPG core proteins. Chain synthesis begins at the region near the plasma membrane that contains between 2 and 4 Ser–Gly GAG attachment sequences. HS biosynthesis involves formation of an initial polysaccharide, composed of alternating D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) units. This polymer is subsequently modified through a series of reactions, requiring both N-deacetylation and N-sulfation of GlcNAc units, epimerization of GlcA to L-iduronic acid (IdoA) residues, and O-sulfation at various positions (reviewed in [11]). From these modifications, enormous structural heterogeneity in HS structure can be produced and several classes of GAG chains can be generated. In the past few years, some of the enzymes and a sugar transporter that participate in HS biosynthesis have been identified and genetically studied in *Drosophila* (Figure 2 and see below).

The role of HSPGs in Wingless signaling

Drosophila Wingless (Wg) belongs to a family of highly conserved proteins — the Wnt family — which encodes secreted glycoproteins that control cell proliferation and

Figure 1



A schematic depiction of cell-surface heparan sulfate proteoglycans (HSPGs). Syndecan and glypicans (e.g. *dally* and *dlp*) represent the two main cell-surface HSPGs and are membrane-associated proteins. (a) The syndecan core protein is a transmembrane protein that bears HS chains distal from the plasma membrane. (b) The glypican core proteins are covalently linked to a phosphatidylinositol (GPI) in the outer leaflet of the plasma membrane. The HS chains on the glypicans are located adjacent to the plasma membrane in the extended region.

differentiation during development via intercellular signaling. Wg participates in a number of developmental processes. In particular, during embryonic development, Wg plays a critical role in patterning the entire segmental unit to specify ventral naked cuticle and in generating cell-type diversity in the epidermis. In addition, Wg is required for other developmental processes, including segmental patterning of the midgut epithelium, development of the Malpighian tubules, formation of the Stomatogastric nervous system (SNS), specification of a subset of neuroblasts, and imaginal disc patterning [12,13].

Enzymes involved in HSPG biosynthesis are required for Wg signaling

The first genetic evidence for the involvement of HS biosynthesis enzymes in Wg signaling came from the characterization of mutations in the *sugarless* (*sgl*) gene, which encodes a *Drosophila* homologue of uridine diphosphoglucose (UDP) dehydrogenase, an enzyme which is required for making UDP-glucuronate from UDP-glucose. *sgl* mutations were identified in screens for mutants that exhibit, in the absence of both maternal and zygotic gene activities, *wg*-like segment polarity phenotypes [14–16]. In addition, *sgl* mutations were identified in a screen for mutants that enhance a weak *dishevelled* viable adult phenotype [17].

The Wg signaling pathway is mediated by several downstream components, which include *Drosophila Fz2* (*DFz2*), *dishevelled* (*dsh*) and *armadillo* (*arm*). Loss-of-function mutations in these genes result in a cuticle pattern with a lawn of denticles. Also, because of the existence of a feedback mechanism, mutations that block Hh signaling are associated with a loss of *wg* expression, and thus resemble *wg* mutants.

In addition to *sgl*, mutations in two additional genes, *sulfateless* (*sfl*), a homologue of HS *N*-deacetylase/*N*-sulphotransferase required for the modification of HS GAG [18*] and *fringe connection* (*frc*), a UDP-GlcA transporter, were identified in screens for mutants that exhibit a *wg*-like segment polarity

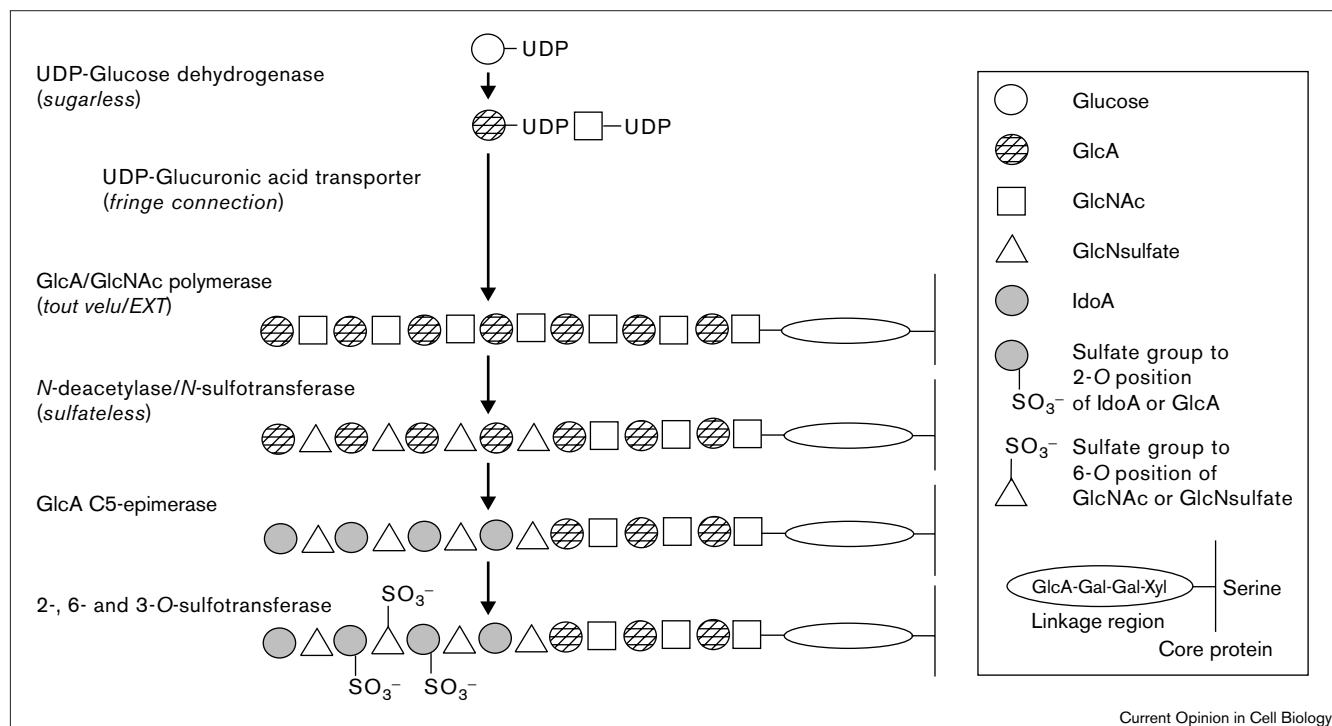
phenotype (E Selva *et al.*, unpublished data). It has been proposed that *frc* activity is required to transport the GlcA produced by *sgl* in the cytosol into the Golgi, where it subsequently serves as a substrate for HS biosynthesis.

In the absence of both maternal and zygotic expression of either *sgl*, *sfl* or *frc* gene activities, embryos die with a segment polarity phenotype. The embryonic phenotypes of these mutants are reminiscent of those associated with either *wg* or *hh* mutations, suggesting that these genes are involved in either or both of these signaling pathways. Further analyses of Wg-specific developmental processes revealed a requirement of HS in Wg signaling. For example, in either *sgl* or *sfl* null mutant embryos the formation of the SNS, which depends on Wg signaling but not Hh, is partially impaired. This phenotype is similar to that of the partial loss of Wg signaling, as observed in a weak *arm* mutant background [19]. In support of this model, injection into embryos of heparinase I and II, enzymes that degrade heparin-like GAGs, generates *sgl* phenocopies. Consistent with this, injection of HS rescues *sgl* mutants [15]. *sfl* is also required for Wg signaling in the development of the wing-imaginal discs [18*]. In *sfl* mutant discs, the expression of the Wg target genes *neutralized* (*neu*) and *distalless* (*dll*) are dramatically reduced, indicating a role for HSPGs in both short- and long-range Wg effects during wing-disc development. This dependence of Wg signaling on HS has also been shown in cell-culture assays. Wg signaling can be inhibited by treatment of cells with sodium percholate, a competitive inhibitor that blocks the sulfation of PGs [4].

Core proteins involved in HSPG biosynthesis are required for Wg signaling

Evidence suggests that the two *Drosophila* glypican molecules, Division abnormally delayed (Dally) and Dally-like protein (Dlp), are the protein cores of the HSPGs involved in Wg signaling ([18*,20*]; G-H Baeg *et al.*, unpublished data). Loss of *dally* gene activity, which was analyzed both

Figure 2



Structure of heparan sulfate (HS) chains. The chains are covalently linked to serine residues of the core protein and share a common tetrasaccharide linkage region. HS is synthesized by the addition of

alternating GlcA and GlcNAc residues, producing the precursor chain. This chain is then enzymatically modified by *N*-deacetylation/*N*-sulfation, epimerization and *O*-sulfation, yielding enormous structural heterogeneity.

using hypomorphic *dally* mutants as well as using RNA interference (RNAi) methodology, is associated with segment polarity defects reminiscent of loss of *wg* activity. Further genetic interaction experiments between *dally* and components of the Wg signaling pathway are consistent with a model in which Dally receives, together with the seven transmembrane DFz2 receptor, Wg. Dally is required for formation of the ectopic bristles associated with overexpression of DFz2 in the wing pouch. In addition, the phenotype associated with ectopic expression of a dominant-negative form of DFz2 is enhanced in homozygous *dally* mutants [21]. Finally, ectopic expression of a gain-of-function Arm protein can fully rescue the wing margin defects associated with *dally* mutants. Altogether, these results suggest that the wing margin defects are specific to Wg signaling and that Dally acts upstream of Arm.

Recently, a novel *Drosophila* glypican molecule, Dlp, has been identified. The predicted primary structure of the novel PG has the hallmarks of vertebrate glypican family members. Disruption of *dlp* gene activity using RNAi methodology also results in segment polarity cuticle defects reminiscent of the phenotype exhibited by mutations in *wg*. Interestingly, *dlp* mRNA expression, like *dally*, is similar to the expression pattern of DFz2 in the embryo. Those transcripts are enriched in a segmentally repeated pattern in three to four cells anterior to *wg*-expressing cells. In addition,

ectopic expression of Dlp in the wing imaginal disc results in an increased accumulation of Wg. These data support the model that HS GAG chains of Dally and/or Dlp are involved in Wg reception. Although the detailed molecular mechanism of HS function in Wg signaling remains to be elucidated, these results clearly implicate HSPGs in Wg signal transduction *in vivo*.

The role of HSPGs in FGF signaling

In addition to defects in Wg signaling, transmission of the FGF-dependent signal is also affected in the absence of either *sgl*, *sfl* or *frc* gene activities. FGF extracellular ligands signal through high affinity transmembrane receptor protein tyrosine kinases. Biochemical studies have also identified HSPGs as low affinity FGF coreceptors that facilitate FGF signal transduction [22,23]. Both *sgl* and *sfl* mutant embryos have phenotypes similar to those lacking the functions of the two *Drosophila* FGFRs, Heartless (Htl) and Breathless (Btl) [24]. Htl is required for the dorso-lateral migration of early embryonic mesoderm cells, whereas Btl is required for the migration and determination of certain tracheal cells. These receptors normally transduce their intracellular signals using the conserved Ras/MAP kinase signaling cassette. In both *sgl* and *sfl* null mutants, Htl- and Btl-dependent MAP kinase activation is significantly reduced. HS GAGs may be required to stabilize and facilitate the self-association of FGF molecules

into physiological concentrations. Consistent with an involvement of *sgl* and *sfl* in FGF signaling, overexpression of *Branchless (Bnl)*, which encodes the ligand for Btl or a constitutively activated form of Htl, partially rescues the tracheal cell migration defects and mesodermal cell migration defects in either *sgl* and *sfl* mutants [24].

The role of HSPGs in Hedgehog signaling

HSPGs also play a key role in Hh signaling. Hh is a secreted heparin-binding protein that exerts both short and long range effects in a variety of developmental processes. In the developing wing disc, Hh travels to and acts at a distance of 8–10 cell diameters from the site of its production to induce the expression of its target genes *patched (ptc)* and *dpp* along the anterior/posterior (A/P) boundary of the wing imaginal disc [25,26]. Through an auto-processing event, a cholesterol moiety is added to the amino-terminal (HhN) fragment, following cleavage from a precursor Hh protein, to generate HhNp. HhNp is responsible for all the biological activities of Hh in both flies and vertebrates.

Most of what is known about the role of HSPGs in Hh signaling was obtained from the studies of the gene *tout-velu (ttv)*; which means ‘all hairy’ in French). *Ttv* encodes a type II transmembrane protein that belongs to the *Ext* gene family. *Ext* genes have been implicated in the human exostoses syndrome, characterized by bone outgrowths and higher incidence of bone tumours (chondrosarcomas and osteosarcomas) [27,28]. Biochemical studies suggest that *Ext* proteins encode HS polymerase, which may function as a GlcA transferase and/or a GlcNAc transferase to generate the initial HS polysaccharide precursor [29–31]. Thus, in the absence of *Ttv/Ext* enzymes, the GAG chains are not properly synthesized, resulting in less HS on the protein cores.

A number of results support the model that *ttv* is required for the proper distribution of the membrane-targeted cholesterol-modified Hh molecule through tissues. Results from mosaic analyses have revealed that *ttv* is required in Hh-receiving cells for the movement of Hh from sending to receiving cells [32]. In addition, in the embryo, in the absence of both maternal and zygotic *ttv* activity, both *wg* and *engrailed (en)* expression fades, which is reminiscent of loss of Hh signaling [33•]. Surprisingly, analysis of *ttv* mutants, both in embryos and wing discs, revealed that Hh signaling, but not Wg and FGF signaling via HSPGs-dependent processes, is affected. Consistent with the phenotypic observations, Hh but not Wg, distribution is affected in *ttv* mutants. The apparent specificity of *Ttv* for Hh, but not FGF or Wg, signaling was unexpected because it suggests that *Ttv* may be involved in the biosynthesis of a Hh-specific HSPG. An alternative model is that Hh signaling is more sensitive to a reduction in HSPGs concentration than Wg and FGF signaling, as in the absence of *Ttv* activity, residual HSPGs are present in the cell, presumably owing to the activity of other *Ext* genes [33•].

Recently, a novel segment polarity gene *Dispatched (disp)* was identified [34]. Interestingly, *Disp* acts exclusively in Hh-secreting cells to liberate the cholesterol-tethered form of Hh (HhNp) from either the internal or surface membranes of the cells. Perhaps HhNp is released from *Disp* on Hh-producing cells by interacting with a *ttv*-modified cell-surface HSPG on the adjacent receiving cells, and subsequently, the HSPG will be required to transfer HhNp to more distantly located cells (reviewed in [35]).

The role of HSPGs in Decapentaplegic signaling

Dpp, a *Drosophila* member of the TGF- β /bone morphogenetic protein (BMP) superfamily, is a critical regulator of differentiation and cell division during development. Particularly, these secreted factors pattern tissues in a concentration-dependent manner. Thus, regulating the extracellular distribution of *Dpp* is critical to patterning. In developing discs, a reduction in *Dpp* level increases the defects associated with *dally* mutations in the eye, antenna and genitalia [36]. Furthermore, additional copies of *Dpp* rescue the defects in these tissues. These genetic interactions between *dpp* and *dally* suggest a role for *dally* in *Dpp* signaling.

Surprisingly, there is no evidence from the studies of either *sgl*, *sfl* or *dally* mutants that HSPGs play a role in the embryonic function of *Dpp* in the establishment of dorsal ventral (D/V) polarity. During embryogenesis, *Dally* is involved in Wg signaling, whereas in developing discs it regulates aspects of both Wg and *Dpp* signaling. These findings provide a striking example of how HSPG is used by different pathways in a developmentally regulated fashion. The tissue-specific effects of *Dally* in *Dpp* signaling might be explained by tissue-specific modulation of the *Dally* protein core by HS GAG chains.

Role of sulfotransferases in HSPG specificity

Studies in *Drosophila* provide growing evidence for the specific roles of HSPGs in signaling pathways. The diversity of the HSPGs could be at the level of the GAG chains. Thus, sulfotransferases that modify the GAG are good candidates for defining this specificity. In vertebrates, a number of sulfotransferases are differentially expressed in various tissues [37], suggesting that specific HS GAGs can decorate the cell surface. Recently, a tissue-specific putative sulfotransferase, *Pipe*, that plays a role in the establishment of the D/V embryonic axis has been identified in *Drosophila*. *Pipe* encodes an enzyme similar to the GAG-modifying enzyme heparan sulfate 2-O-sulphotransferase and is expressed in ventral follicle cells during oogenesis [38]. *Pipe* mutant embryos show defects in the establishment of D/V polarity, a process that depends on the ventral activation of the uniformly distributed Toll receptor. The ligand for Toll is encoded by *Spätzle*, which is activated by a proteolytic cascade [39–41]. The analysis of *pipe* suggests that a *Pipe*-modified PG either activates or assembles a protease complex that processes *Spätzle* or concentrates the inactive *Spätzle* at the ventral side of the egg chamber. This facilitates

its processing, leading to active Spätzle. Although it has been demonstrated that *pipe* is involved in the modification of GAGs required for D/V pattern formation, it is not yet known whether the *pipe* gene encodes a sulfotransferase for a HSPG or another class of PG.

Conclusions

During the development of multicellular organisms, the generation of complex patterns is controlled by several key signal molecules. Recent genetic analyses in *Drosophila* have illustrated the critical roles of HSPGs in developmental processes and specific signaling pathways. A number of mutations in genes involved in GAG chain biosynthesis and genes that encode the core proteins exhibit phenotypes reminiscent of the loss of Wg, Hh, Dpp and FGF. Further, the establishment of embryonic D/V polarity is controlled by a putative sulfotransferase.

Although these studies have pointed to the pivotal roles of HSPGs in the regulation of ligand–receptor interactions, as well as their role in regulating the movement of ligands through a field of cells, the precise mechanism by which HSPGs transduce signals still remains to be determined. For example, how do HSPGs form a complex with ligands and/or their functional receptors to transduce extracellular signals? Further, how do HSPGs facilitate movement of ligands through tissues? Further *in vivo* and biochemical analyses of the core proteins, enzymes and transporters involved in HS biosynthesis will most probably provide new insights into the function of HSPGs, as well as the mechanism of tissue-specific action of HSPGs. In addition, a more complete understanding of the structure and biosynthesis pathway of HS will be required to fully analyze the complexity of HSPG function.

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